Indian J. Genet., 79(4) 678-684 (2019)
DOI: 10.31742/IJGPB.79.4.5

R1-nj expression in parental inbreds as a predictor of amenability of maize hybrids to *R1-nj*-based doubled haploid development

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(Received: May 2019; Revised: July 2019; Accepted: September 2019)

Abstract

The current method of doubled haploid (DH) development in maize involves *in vivo* production of haploids using *R1-nj*-based haploid inducer lines that upon use as male render a small fraction of seed in the pollinated female ears haploid. Identification of haploid seed relies on *R1-nj* marker expression in the endosperm and embryo, and the degree of its expression determines efficiency of DH development process. In the present study, *R1-nj* expression in the endosperm was characterized in crosses of CIMMYT's *R1-nj*-based haploid inducer TAILP1 with a set comprising 18 early maturity hybrids and their 23 parental inbreds. Kernel colour inhibition was observed only in a small proportion of the hybrids and inbreds. Comparison of *R1-nj* expression in the hybrids and their parental inbreds revealed a distinct pattern, which may be useful in identifying source populations and/or determining parental constituents for synthesizing source populations with predicted amenability to doubled haploid development using *R1-nj*-based haploid inducers. However, deviation from the pattern was noted in hybrids involving inbreds with higher degree of colour inhibition, which suggests complex nature of *R1-nj* phenotype expression and necessitates further investigation involving larger sets of germplasm for dissecting the role of maternal and paternal genetic factors in determining *R1-nj* phenotype expression. The hybrids found exhibiting complete kernel anthocyanin expression in present study can be used directly as source populations for DH development using *R1-nj* based haploid inducers. Besides, since the inbreds used in the study have originated from and/or are accessible to CGIAR/NARS maize breeding programmes, the information on their kernel anthocyanin expression can be helpful in selection of source populations or generating new source populations amenable for DH development using *R1-nj* based haploid inducers.

Key words : Maize, haploid inducer, *R1-nj* expression, doubled haploid, amenability

Introduction

Doubled haploid (DH) development has emerged as a promising method for rapid generation of completely homozygous lines for accelerating hybrid development process in maize. The current method of DH development in maize involves *in vivo* production of haploids using *R1-nj* based haploid inducer lines that upon use as male render a small fraction of seed in the female ears haploid (Prasanna et al. 2012). The haploid seeds are subsequently diploidized to obtain completely homozygous lines. Identification of haploids at seed stage is based on phenotypic expression of *R1-nj* (Navajo phenotype), which is characterized by purple colouration in the aleurone layer on the crown region of the endosperm and the scutellum of the embryo (Nanda and Chase 1966; Greenblatt and Bock 1967). *R1-nj* is an allele of *R1* regulatory gene which regulates kernel anthocyanin biosynthesis and its expression requires other structural genes involved in anthocyanin biosynthesis pathway (Chase 1969; Geiger 2009). The use of this method, however, is limited by the presence of dominant anthocyanin inhibitor genes (*C1-I*, *C2-Idf* and in-1D) that inhibit anthocyanin biosynthesis in maize endosperm and embryo (Coe et al. 1988; Stinard and Sachs 2002). Depending on the homozygosity or heterozygosity of the inhibitor alleles in the source population, *R1-nj* expression may be completely inhibited in all kernels or may segregate for color expression among the kernels in an induction cross. This reduces the efficiency of haploid identification based on crosses of source germplasm with the *R1-nj*-based haploid inducers (Chaikam et al. 2015) and may altogether render potential source germplasm unsuitable for DH

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Published by the Indian Society of Genetics & Plant Breeding, A-Block, F2, First Floor, NASC Complex, IARI P.O., Pusa Campus, New Delhi 110 012; Online management by www.isgpb.org; indianjournals.com

development using *R1-nj*-based Haploid Inducer Lines (HILs). DH development in maize using haploid inducers involves considerable time and resources. At CIMMYT, for producing an average 200 DH lines per source population, at least 150 plants of each source population are raised for generating induction crosses (Chaikam et al. 2012). Use of a source germplasm in an induction cross without prior knowledge of its 'Navajo' expression behaviour may result in inefficiency in DH development process if the source germplasm happens to carry colour inhibitor genes. Making test induction crosses for assessing amenability of a source germplasm (before undertaking large scale DH development with it) requires additional crop season besides being labour and resource intensive. Red root marker-based inducer lines have been developed (Chaikam et al. 2016) and seedling traits identified for classification of maternal haploids (Chaikam et al. 2017) in germplasm with inhibitor gene/s. These methods, however, have their own limitations in being labour- and resource-intensive as both methods require germination of a large number of seeds from induction crosses for haploid classification. Chaikam et al. (2015) developed *C1-I* allele-specific diagnostic markers (a combination of two gene-specific markers–8 bp *C1-I* InDel and *C1-I* SNP) for assaying *R1-nj* expression to facilitate identification of germplasm with dominant colour inhibitor genes and predict amenability of a source germplasm to haploid induction using *R1-nj*-based haploid inducers without having to undertake test induction crosses. The application of this method also is limited by the fact that both kernel anthocyanin biosynthesis and inhibition involve more than one gene (Chase 1969; Geiger 2009; Coe et al. 1998; Stinard and Sachs 2002). Therefore, till such time as simpler methods for predicting amenability to DH development using *R1-nj*-based HILs with high accuracy are available, undertaking test induction crosses with the source populations themselves or with the parental constituents of source populations may be an effective way for identifying amenable germplasm/populations. Especially for maize breeding programmes that are at early stages of DH programme initialization and rely majorly on indigenous or in-house germplasm. However, as there are no published reports of comparison of *R1-nj* phenotype expression in hybrids and their parental inbreds, the present study was undertaken with the objective of assessing if, and how, the parental inbreds and their hybrids behave with respect of kernel anthocyanin colour expression in crosses with *R1-nj*-based haploid inducers, and by that means try to identify pattern, if any exists, that may be useful in prediction of amenability of source populations to *R1-nj*-based haploid inducer lines.

Materials and methods

A set of 41 maize genotypes comprising 18 early maturing hybrids and their 23 parental inbreds developed at ICAR-VPKAS, Almora, was used for the study (Table 1). A single 3m long row of each inbred and hybrid was sown in *kharif* 2018 at ICAR-VPKAS, Experimental Farm, Hawalbagh (1250 m amsl, latitude 29º362 N, longitude 79º402 E). The row-to-row spacing was kept at 60 cm and plant-to-plant spacing was maintained at 25 cm so as to have a final stand of 12 plants in each row. *R1-nj*-based tropicalized haploid inducer TAILP1 (EC805127), developed at CIMMYT in collaboration with University of Hohenheim and provided by ICAR-Indian Institute of Maize Research, Ludhiana, was used as the pollinator for the inbred-hybrid set (Fig. 1.).

Table 1. Parental inbreds and hybrids used in the study

S.No.	Hybrid	Parentage	S.No.	Hybrid	Parentage
1.	VMH 9	CM 212 x CM 145	10.	VMH 45	V 373 x V390
2.	Vivek QPM 9	VQL 1 x VQL 2	11.	VMH 47	V 373 x V391
3.	VMH 21	CM 212 x V 341	12.	CMVLSC 1	VSL 16 x VSL 4
4.	VMH 23	V 351 x V 341	13.	FH 3754	V 412 x V 433
5.	VMH 25	V 341 x V 346	14.	FQH 106	VQL 1 x VQL 373
6.	VMH 27	V 335 x V 345	15.	VMH 51	V405 x V409
7.	VMH 33	CM 212 x V 372	16.	VMH 53	V407 x V409
8.	VMH 39	V 373 x CM212	17.	CMVL 55	V 405 x V407
9.	VMH 43	V 373 x V 341	18.	FH 3703	V 400 x V 430

Fig. 1. Plants and mature ears of haploid inducer line TAILP1 at ICAR-VPKAS, experimental farm, Hawalbagh

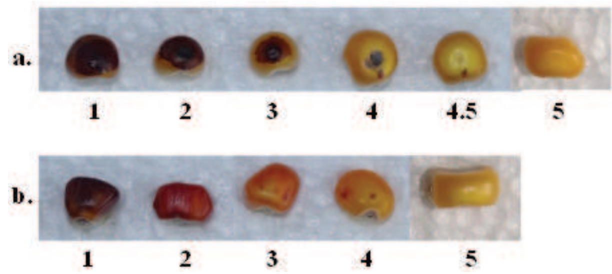

Fig. 2. Scores assigned to seeds for a = area marked and b = intensity of colour

Fig. 3. Variation for *R1-nj* expression in a = inbreds and b = hybrids

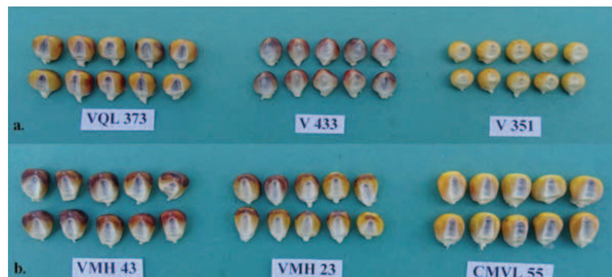

Fig. 4. Variation for *R1-nj* expression in scutellum in a = inbreds b = hybrids

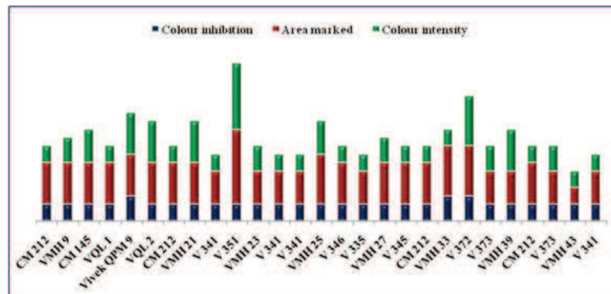

Fig. 5a. Expression pattern of *R1-nj* in parental inbreds and their hybrids (1-5 scale)

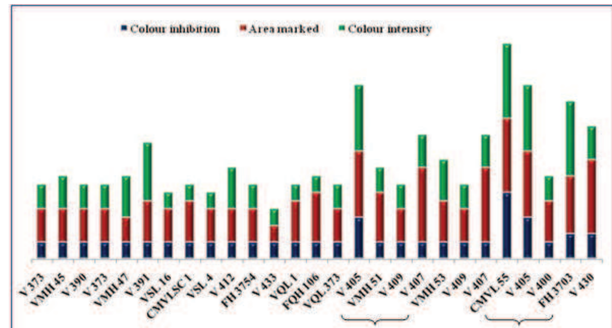

Fig. 5b. Expression pattern of *R1-nj* in parental inbreds and their hybrids (1-5 scale)

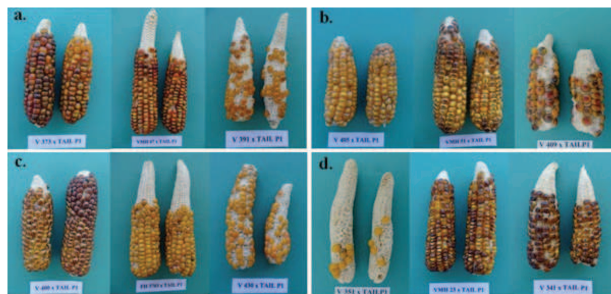

Fig. 6a-d. Differential *R1-nj* expression in inbred-hybrid combinations

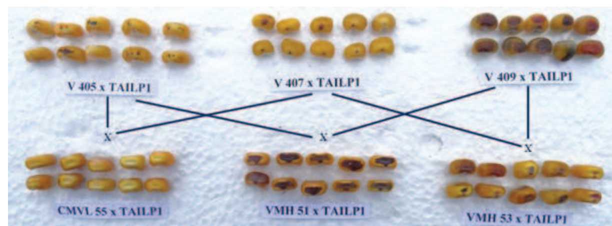

Fig. 7. *R1-nj* expression on endosperm in hybrids VMH 51, VMH 53, CMVL 55 and their parental inbreds

Since TAILP1 is a shy pollen producer at Hawalbagh location, a sufficiently large population (20 rows of 3m long each) of TAILP1 was raised to have sufficient pollen for pollinating the inbred-hybrid set. TAILP1 takes 48-50 days to anthesis at Hawalbagh location, whereas the range of silking in the inbreds and hybrids used was 51-58 days, TAILP1 was therefore planted on two dates separated by 7 days to ensure synchrony in flowering with the inbreds and hybrids. At flowering, individual plants in the inbreds and hybrids rows were pollinated manually with bulk pollen of TAILPI. The pollinated ears from each row of

the inbred-hybrid set were harvested separately at physiological maturity and dried under sun. Five pollinated ears in each inbred and hybrid were selected for recording observations. From each selected ear, 20 kernels were randomly sampled for recording observations on inhibition of colour marker expression, area marked and colour intensity on the endosperm on a scale of 1-5 following the method given by Chaikam et al. (2017) with some modifications (Table 2, Fig. 2).

Table 2. Classification of genotypes

Parameter		Score				
		1	2	3	4	5
Colour inhibition	Proportion of kernels expressing *R1-nj* marker	100%	75%	50%	25%	0%
Area marked*	Extent to which the endosperm expresses *Navajo* phenotype	Entire endosperm	Upper portion of endosperm	Only crown region	Only central portion of crown region	Complete absence of expression
Intensity of pigmentation	Intensity of anthocyanin colour expression on the endosperm	Deep purple/ red	Purple/red	Light red/ orange	Light orange	Visually undetectable

*score of 4.5 was assigned to kernel with anthocyanin expression confined to the silk attachment region

While Chaikam et al. (2017) assigned a score of 2.5 to indicate colour marker expression in nearly 50 per cent of the kernels, we used a score of 3.0 for the same. Kernels up to a score of 4.5 for area marked were counted as having colour marker expression. Based on the proportion of kernels expressing *R1-nj*, the inbred-hybrid set was categorized into three groups: 1. Full colour expression (score 1-2), 2. Segregating for colour expression (score 2.5-4) and 3. Complete colour inhibition (score>4). It is pertinent to mention here that variation for kernel anthocyanin colour expression within and among ears of the same genotype is common, therefore, observer's discretion is often required in classifying seeds into different categories.

Results

Variation for R1-nj marker expression on endosperm

Forty one maize genotypes comprising 18 hybrids and their 23 parental lines were crossed with TAILP1 and observations on kernel anthocyanin pigmentation were recorded at maturity. The summary of observations on *R1-nj* expression on the endosperm in the germplasm set is presented in Table 3. For endosperm colour inhibition, the inbreds fell in 'full kernel colour expression' to 'segregating for colour expression' range (score 1-4), whereas in the hybrids, the range extended up to 'complete colour inhibition' (score>4) due to presence of visually undetectable colour expression in one of the hybrids. For area marked and intensity of colour, the germplasm set showed range from 'full kernel colour expression' to 'complete colour inhibition'.

Table 3. *R1-nj* expression in hybrids and inbreds (1-5 scale)

	Hybrids		Inbreds	
	Range	Average	Range	Average
Colour inhibition	1.0-4.0	1.3	1.0-2.5	1.1
Area marked	1.0-4.5	2.4	1.0-4.5	2.6
Intensity of colour	1.0-4.5	2.0	1.0-4.0	1.8

R1-nj expression in the germplasm set exhibited wide variation for endosperm colour inhibition, area marked and intensity of colour (Fig. 3a,b). In majority of the genotypes, colour expression was limited to the crown region, which is a typical characteristic of *R1-nj* expression. Among hybrids, all except CMVL 55 showed full kernel colour expression. Pigmentation was most intense in VMH 43 which also had highest area marked. Among inbreds, none showed complete colour inhibition, while one (V 430) segregated for colour expression. In V 430 and V 407, colour expression was represented by a small coloured dot at silk attachment. Highest colour intensity and area marked was observed in kernels of inbred V 433. Overall, with

respect to *R1-nj* expression on endosperm, 94.4% hybrids and 95.7% inbreds exhibited full kernel colour expression (Table 4).

Table 4. Categories of *R1-nj* expression in hybrids and inbreds based on proportion of kernels expressing the phenotype

Type of germplasm	No.	Full kernel colour expression (%)	Segregation for colour expression (%)	Complete colour inhibition (%)
Hybrids	18	94.4 (17)	0.0	5.6 (1)
Inbreds	23	95.7 (22)	4.3 (1)	0.0
Total	41	95.1 (39)	2.4 (1)	2.4 (1)

*Figures in parenthesis indicate number of genotypes

R1-nj expression on embryo

Variation for *R1-nj* expression on the embryo was observed in the germplasm set. Complete colour expression was observed in all the inbreds except V 351 (Fig. 4a), which exhibited lower intensity of colour but was nonetheless easily detectable visually. Among hybrids, all hybrids showed full colour expression on embryo that included CMVL 55, which showed colour inhibition on endosperm (Fig. 4b).

R1-nj expression in Inbred-Hybrid combinations

Comparison of *R1-nj* expression of parental inbreds with their corresponding hybrids revealed a distinct pattern (Fig. 5a,b). Barring two exceptions (indicated by braces in Fig. 5b), kernel colour expression of hybrids mostly matched that of their parental inbreds. The exceptions were represented by VMH 51 and CMVL 55 that share V 405 as the common parent. Other hybrids that showed deviations, though of a smaller magnitude, from the observed pattern were Vivek QPM 9, VMH 33 and FH 3703.

The pattern observed in inbred-hybrid combinations for colour inhibition was observed for area marked as well, except for VMH 43 which showed deviation from the pattern by exhibiting larger area marked compared to its parental inbreds V 373 and V 341. Smaller deviations were observed for VMH 51, CMVL 55, FH 3703 and FH 3754. For intensity of colour, many hybrids, namely, VMH 51, CMVL 55, FH 3703, VMH 21, VMH 33 and VMH 39, showed prominent deviation from their parental inbreds, and a distinct pattern as observed for colour inhibition and area marked was not observed. Considering all the three endosperm parameters together, it was observed that parental inbreds with full kernel colour expression imparted the same expression to their hybrids. The expression of most of the hybrids with one parent with full kernel colour expression, higher area marked and colour internsity, and other with incomplete color expression tended to be towards the parent with full kernel expression (Fig. 6a,b,d). Deviation was observed in case of hybrid FH 3703 (Fig. 6c) which exhibited lower area marked and colour intensity than parental inbred V 400, and hybrid CMVL 55 which showed complete colour inhibition (Fig. 7.)

Further, comparison of inbreds V 405, V 407 and V 409 with their corresponding hybrids revealed a complex interaction between parental inbreds for *R1-nj* endosperm expression. VMH 53 (V 407/V409) matched its parental inbreds in kernel colour expression, whereas V 405 behaved differently in CMVL 55 (V 405/V 407) and VMH 51 (V 405/V 409) (Table 5 & Fig. 4). While kernel colour inhibition of V 405 was suppressed in VMH 51 in interaction with the male parent V 409, colour inhibition was pronounced in CMVL 55 which has a colour expressing inbred V 407 as the female parent. The hybrid VMH 53 with colour expressing inbreds V 407 and V 409 as female and male parent, respectively, exhibited complete kernel anthocyanin colouration.

Discussion

Doubled haploid technology is increasingly being adopted in maize breeding programmes to shorten inbred generation time and accelerate hybrid

Table 5. *R1-nj* expression in inbreds V 405, V 407, V 409 and their hybrids (1-5 scale)

	V 405	V 407	V 409	CMVL 55 (V 405/V 407)	VMH 51 (V 405/V 409)	VMH 53 (V 407/V 409)
Colour inhibition	2.5	1.0	1.0	4.5	1.0	1.0
Area marked	4.0	4.5	2.0	4.5	2.0	2.5
Intensity of pigmentation	4.0	2.0	1.5	4.5	1.0	2.5

development process. *R1-nj*-based haploid inducer lines are currently the most employed inducers for haploid induction in maize. Haploid kernel identification in maize relies on expression of 'Navajo' phenotype in the seeds from induction crosses, the expression of which shows wide variation depending upon the background of the source germplasm (Chaikam et al 2015). Incomplete expression of the phenotype reduces efficiency of haploid selection and, consequently, of the entire DH development process. Prior evaluation of amenability of the source gemplasm/ constituents of the source population to DH development using *R1-nj* based inducers is important before taking up large scale DH development programme with them. In the present study, among a set of 41 genotypes comprising early maturity hybrids and their parental inbreds (derived from pedigree crosses as well as populations of national and international origin, including those of CIMMYT), 95.1% genotypes expressed full kernel colour on endosperm in the induction crosses with *R1-nj* based haploid inducer TAILP1, suggesting their amenability to DH development. Segregation for *R1-nj* expression was observed in 2.4% genotypes, whereas 2.4% showed complete colour inhibition. Chaikam et al. (2015) assayed a large germplasm set comprising CIMMTY derived maize inbred lines and land race accessions from the CIMMYT Gene Bank with molecular markers and reported complete colour inhibition in 4% of the populations and segregation of *R1-nj* expression in more than 40%, indicating that it is not possible to identify all the haploids induced in these populations. Segregation for the *R1-nj* expression in a breeding population is possible when one or more inbred lines used in deriving the population possess colour inhibiting gene/s (Chaikam et al. 2015). Roop Kamal (2017) also reported absence of *R1-nj* expression in two out of four crosses studied.

Comparison of parental inbreds with their hybrids for *R1-nj* expression on endosperm revealed that hybrids of parental inbreds with full kernel colour expression also had kernels with full colour expression, suggesting that constituents with full kernel colour expression are highly likely to produce populations fully amenable to DH development using *R1-nj*-based inducers. In majority of hybrid combinations where one parent expressed full kernel colour and the other expressed incomplete colour, expression in their hybrids was towards full kernel colour. Such hybrids were amenable to *R1-nj*-based haploid classification despite one parent expressing incomplete kernel colour. This suggests that endosperm colour expression in the parental inbreds may serve an indicator for prediction of amenability of their hybrids as source populations for DH development using *R1-nj*-based inducers. It also infers that inclusion/ exclusion of potential germplasm as constituents of a source population may be based on its *inter se* (with other constituents) rather than *per se* performance with respect to *R1-nj* endosperm colour expression. However, the differential interaction of inbred V 405 observed in the investigation necessitates analysis of individual combinations involving inbreds with colour inhibition. Since none of the hybrids used in the study involved both parental inbreds with complete colour inhibition, kernel pigmentation expression in hybrids with both parental inbreds with complete colour inhibtion could not be ascertained. Study of kernel endosperm colour expression in a larger set of hybrids/ populations with colour inhibition in parental inbreds could provide interesting insights into the interactions among maternal and paternal genetic factors for determining kernel anthocyanin pigmentation. It is important to note that between area marked and intensity of colour, the latter is more helpful in classification of seed as higher colour intensity allows visual detection of even very small anthocyanin pigmentation marks on the endosperm (like the mark at the silk attachment region), whereas higher area marked with lower colour intensity may often lead to misclassification of seeds.

In conclusion, about 95.1 per cent germplasm expressed full anthocyanin kernel pigmentation. *R1-nj* inhibition was found in less than 5% inbreds and hybrids. Full kernel colour expression in both parental inbreds assures complete *R1-nj* expression in hybrids, however, the same is not likely to hold true for all combinations comprising constituents with colour inhibition gene/s. The hybrids found expressing complete kernel anthocyanin expression in this study can be used directly as source populations for DH development using *R1-nj* based haploid inducers. Besides, the information on kernel anthocyanin expression of the inbreds can be helpful in selection of source populations or generating new source populations amenable for DH development using *R1-nj* based haploid inducers.

Authors' contribution

Conceptualization of research (RKK, AP); Designing of the experiments (RKK, AP); Contribution of experimental materials (RKK, AP); Execution of field/

lab experiments and data collection (RKK, VP); Analysis of data and interpretation (RKK, AP); Preparation of manuscript (RKK, AP).

Declaration

The authors declare no conflict of interest.

Acknowledgments

The authors gratefully acknowledge the financial support received from ICAR-National Agricultural Science Fund, New Delhi and ICAR-VPKAS, Almora. The *R1-nj*-based tropical haploid inducer TAILP1 used in this study was provided by ICAR-Indian Institute of Maize Research, Ludhiana.

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